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TANELI TIRKKONEN
Porcine Mycobacteriosis Caused by Mycobacterium avium subspecies hominissuis

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Porcine mycobacteriosis caused by Mycobacterium avium subspecies hominissuis

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Porcine mycobacteriosis caused by Mycobacterium avium subspecies hominissuis

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Dedicated to my dear wife Ilona
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Abstract

More than 150 species of mycobacteria are described, most being opportunistic pathogens and all representing a risk for human and animal health. Human infections derived from environmental mycobacteria are increasing in both industrialized and developing countries. The most susceptible groups are children, the elderly and those, including animals, with immunocompressive conditions. Drug therapy for mycobacteriosis is difficult and not always successful. Infections caused by drug-resistant mycobacteria can be life threatening also for healthy adults and thus represent a real risk for humans. Environmental mycobacterial infections of pigs are usually without clinical signs and the lesions are mainly detected at slaughter. *Mycobacterium*-infected pork can pass for human consumption due to the poor sensitivity of visual meat control at slaughterhouses, and mycobacteria in pigs also cause economic losses due to condemnation of carcasses. The main challenge is represented by evaluation of the hygiene risk associated with using mycobacteria-contaminated pork.

Most environmental mycobacteria species have been isolated from sources such as water, swimming pools, soil, plants and bedding material. In our study mycobacterial growth in piggeries was identified in all bedding materials, sawdust, straw, peat and wood chips in most cases, and water and food samples in many cases, and only occasionally in dust and on wall surfaces. The maximum number of mycobacteria was almost 1 billion (10⁹) per gram of bedding, which is close to the maximum concentration in any growth media. Mycobacteria can multiply in piggeries and contaminate feed and water. Isolation of mycobacteria from pig faeces can be considered an indicator for risk of human infection.

Environmental mycobacteriosis in humans and pigs is mainly caused by *M. avium* subsp. *hominissuis*. There is little evidence of direct transmission from animals to humans, but particular strains can be recovered from both humans and pigs. In our studies, identical mycobacteria RFLP and MIRU-VNTR fingerprints of porcine and human origins were evident. Interspecies clusters were more common than intraspecies clusters using both methods. Therefore, we concluded that pigs act as a reservoir for virulent *M. avium* strains and the vector for transmission of infections in humans to pigs, and *vice versa*, may have an identical source of infection.

Culturing mycobacteria is the gold standard for diagnosis, but detection of environmental mycobacteria based on cultivation and biochemical methods can take several weeks. Culture-independent, rapid and accurate techniques for detecting mycobacteria in food and feed chains are urgently needed. In this work we developed a rapid and accurate real-time quantitative PCR for detecting environmental mycobacteria in bedding materials and pig organs.
Conclusion:
Mycobacteria can multiply in bedding materials and the consequent heavy contamination can cause simultaneous infections in pigs. Mycobacterial DNA was found in pig organ samples, including those without lesions, and similar strains were found from humans and pig organ samples, which suggests that mycobacteria can be transmitted between humans and pigs.
List of Original Publications


The author's contribution

Paper I: TT planned and performed the relevant sampling procedure and participated in writing the paper, particularly regarding veterinary medicine and zoonotic aspects.

Paper II: TT performed strain selection for the porcine originating strains for the RFLP typing, interpreted the results and wrote the paper together with the co-authors.

Paper III: TT participated in the study design, sampling, analysis and interpretation of the results, TT wrote the paper and was the corresponding author.

Paper IV: TT participated in the study design, sampling, analysis and interpretation of the results, TT wrote the paper and was the corresponding author.

Abbreviations

AIDS acquired immune deficiency syndrome
DI discriminatory index
DNA deoxyribonucleic acid
DSM Deutsche Sammlung von Mikroorganismen
ELISA-test Enzyme-Linked Immunosorbent Assay
EXP exponent
IS1245 insertion sequence 1245
IS 1245 RFLP IS 1245 Restriction Fragment Length Polymorphism fingerprinting
MAA Mycobacterium avium subsp. avium
MAC Mycobacterium avium complex
MAH Mycobacterium avium subsp. hominissuis
MIRU genetic interspersed repetitive units of Mycobacteria
NTM nontuberculous mycobacteria
PCR polymerase chain reaction
qPCR quantitative polymerase chain reaction
RNA ribonucleic acid
rRNA ribosomal ribonucleic acid
spp. subspecies (plural)
subsp. subspecies
VNTR typing Variable-Number Tandem-Repeat marker typing
1. Literature Review

1.1. Description of the mycobacteria

The genus *Mycobacterium* comprises over 150 species of aerobic, non-spore-forming, non-motile, rod-shaped acid-fast bacilli. The mycobacteria include diverse species, ranging from environmental saprophytes and opportunistic invaders to obligate path-ogens. *M. africanum, M. bovis, M. canettii, M. caprae, M. microti, M. pinnipedii, M. tuberculosis, M. leprae* and *M. lepraemurium* are obligate pathogens of humans and animals (Portaels 1995, Vaerewijck et al. 2005). Although some pathogenic mycobacteria exhibit a particular host preference, they can occasionally infect other species. Mycobacteria can multiply intracellularly and diseases in humans and animals are usually chronic granulomatous and progressive infections (Hibiya et al. 2011, Koh et al. 2002). Obligate pathogens, shed by infected animals, can also survive in the environment for extended periods (Portaels 1995, Vaerewijck et al. 2005).

1.1.1. Environmental mycobacteria

Environmental mycobacteria are a heterogeneous group of slow-growing species that include saprophytes and opportunistic pathogens (El-Sayed et al. 2013, Portaels 1995, Salem et al. 2012, Vaerewijck et al. 2005). Glycopeptidolipids in cell walls render mycobacteria hydrophobic and resistant to adverse environmental influences and dis-infectants, including chlorine. Some *Mycobacterium* spp. are resistant to, and even multiply at, high temperatures (Vaerewijck et al. 2005). The lipid-rich layer of the cell wall increases biofilm formation in some mycobacteria species (Freeman et al. 2006, Johansen et al. 2009, Recht et al. 2000, Recht et al. 2001, Yamazaki et al. 2006). The ability to form biofilms is linked with virulence and resistance in bacteria (Carter et al. 2003, Johansen et al. 2009). Mycobacteria have been isolated from various environments, including soil, water, aerosols, protozoa, deep litter and fresh vegetation from all over the world (Biet et al. 2005, Eisenberg et al. 2010). Opportunistic environmental mycobacteria can cause tuberculous lesions and disseminate infections in humans and animals (Kunze et al. 1992). Human infections due to environmental mycobacteria are increasing in both industrialized and developing countries. The most susceptible risk groups are children, the elderly and those, including animals, with immunosuppressive conditions (Falkinham 1996, Hiller et al. 2013, Vaerewijck et al. 2005). Drug therapy of mycobacteriosis is difficult and not always successful. Infections caused by drug-resistant mycobacteria can be life threatening also for healthy
adults and thus they represent a relevant risk for humans (Eriksson et al. 2001, Hiller et al. 2013, Nylen et al. 2000). Generalized disease in birds and poultry (Pavlic et al. 2000), pigs (Eisenberg et al. 2012), cattle (Möbius et al. 2006), cats and dogs (Thorel et al. 2001), horses, foxes, cervids, game (Moser et al. 2011) small rodents, insectivores (Fischer et al. 2000) and other species caused by members of the Mycobacterium avium complex have been reported (Pavlik et al. 2005, Thorel 1997).

1.1.2. Porcine-related mycobacteria

*Mycobacterium avium* subsp. *hominissuis* belongs to the *Mycobacterium avium* complex and is the most common environmental mycobacterium in infections of humans and pigs (Agdestein et al. 2011, Agdestein et al. 2012, Domingos et al. 2009, Iwamoto et al. 2012, Johansen et al. 2007, Koh et al. 2002, Mijs et al. 2002, Pavlik et al. 2003, Stepanova et al. 2012). The condition of the host can differ between humans and swine: human hosts often have the infection in their lungs (Ashford et al. 2001, Jarzembsowski et al. 2008, Johansen et al. 2009) whereas, pigs are infected usually through oral ingestion. Tubercles can usually be found in the retropharyngeal, submaxillary and cervical lymph nodes of pigs (Matlova et al. 2005, Thorel et al. 2001). Infections in aborted foetuses and in the genital organs of pigs, and decrease in growth rate as well as increased mortality, have been reported (Bille et al. 1973, Eisenberg et al. 2012, Wellenberg et al. 2010). Hepatic lesions are observed in systemically infected pigs. However, mycobacterial pathogenesis is poorly understood (Hibiya et al. 2008, Hibiya et al. 2010). Johansen et al. 2009 speculated that pigs could become infected only when a large infective dose of *M. avium* strains occurs in their living environment. Mycobacteria in a single pig were reported to be multiple variants because mycobacteriosis is of environmental origin (Eisenberg et al. 2012, Wellenberg et al. 2010). In pigs *M. avium* infections can be persistent without any clinical signs, but may nonetheless represent economic losses for the farmer because meat from infected animals is considered unsuitable for human consumption and is condemned (Pavlik et al. 2003). During the last decade, the prevalence of *M. avium* subsp. *hominissuis* in slaughtered pigs increased worldwide (El-Sayed et al. 2013, Möbius et al. 2006). There is also a recent study of *Mycobacterium bovis* in domestic free range pigs in Spain (Cardoso-Toset et al. 2017), but so far there is very limited knowledge on this theme from North-European pigs. Prevalence of mycobacterial-like lesions in Finnish slaughter pigs in the period 1998-2012 is shown in Fig. 1.
Figure 1. Prevalence of mycobacterial-like lesions in Finnish slaughter pigs during 1998-2012. Bars illustrating cumulative annual slaughter pigs and the line % mycobacteria. Finnish Food Safety Authority Evira meat control results database 2013.

1.2. Disease risk of mycobacteria from an epidemiological viewpoint

It has been suggested that drinking water may be an important reservoir of infective mycobacteria, especially for humans (Aronson et al. 1999, Falkinham III 1996), whereas bedding material has proved to be an important source of porcine infections (Alvarez et al. 2011, Matlova et al. 2004, Matlova et al. 2005). Mycobacterial infections have a zoonotic character and similar types of mycobacteria strains have been found in pigs and humans (Komijn et al. 1999, Möbius et al. 2006). It cannot be ruled out that mycobacterial infection of humans may originate from piggeries (Komijn et al. 1999, Komijn et al. 2000, Möbius et al. 2006). Contaminated faecal material in bedding and mycobacteria in raw pork represent potential food safety hazards (Alvarez et al. 2011, Komijn et al. 1999, Matlova et al. 2005, Matlova et al. 2004, Möbius et al. 2006, Johansen et al. 2014).

More knowledge about the modes of transmission in both animals and humans is required for the control of mycobacterioses (Agdestein et al. 2012, Biet et al. 2005, Thegerström et al. 2005). New methods for the identification, genetic profiling, and rapid real-time quantification of environmental mycobacteria are needed to trace environmental reservoirs of human and animal mycobacteriosis and for assessing the risk they may represent.

### 1.3. Detection and identification of mycobacteria

Acid-fast staining is used to differentiate mycobacteria from other bacteria. In pigs, acid-fast bacilli can be found from caseous malformations in lymph nodes, kidneys, liver and spleen (Eisenberg et al. 2012, Hiller et al. 2013, Van Inger et al. 2010, Offermann et al. 1999), but also from *Rhodococcus equi* and infections due to other acid-fast bacilli (Dvorska et al. 1999, Eisenberg et al. 2012, Hiller et al. 2013, Komijn et al. 2007, Pate et al. 2004). However, mycobacteria can be present in porcine lymph nodes without any visible lesions (Dvorska et al. 1999, Hiller et al. 2013). Differentiation of pathogenic mycobacteria relies on cultural characteristics, biochemical tests, animal inoculation, chromatographic analyses and molecular techniques. In addition, mycobacteria associated with opportunistic infections can be differentiated on the basis of pigment production, optimal incubation temperature and growth rate. Pathogenic mycobacteria grow slowly on solid media and colonies are not evident until cultures have been incubated for at least three weeks (Fig. 2).
In contrast, the colonies and growth in broth of rapidly growing saprophytes are visible within days (Fig. 3 A & B).
However, identification of the members belonging to the *M. avium* complex using biochemical testing can take up to several weeks (Slana et al. 2010, Springer et al. 1996). Some mycobacterial isolates cannot be identified using biochemical differentiation because their biochemical profiles are very difficult to interpret (Gunn-Moore et al. 1996).

Culturing mycobacteria is the gold standard of diagnosis, but molecular tests are also used (Agdestein et al. 2011, Inderlied et al. 1993, Thorel et al. 2001, Turenne et al. 2007). DNA probes, complementary to species-specific sequences of rRNA, are commercially available for identifying some mycobacterium species. Nucleic acid amplification procedures, including PCR, were and are being developed for the detection of mycobacteria in environmental and tissue samples (Khan et al. 2004, Kox et al. 1995, Pakarinen 2008, Shrestha et al. 2003, Talaat et al. 1997, Telenti et al. 1993) and DNA restriction endonuclease analyses (DNA fingerprinting) have been used in epidemiological studies over recent decades by several authors (Bauer et al. 1999, Collins et al. 1994, Johansen et al. 2007). However, these techniques are laborious, expensive or not suitable and not sensitive enough for mycobacteria.
1.4. Current methods and recommendations in pork meat control

European law (EU/854/2004) describes the procedure for meat inspection at slaughter, including palpation and incision of the lymph nodes, including the procedures for detection of porcine mycobacterial-like infections (Fig. 4, Fig. 5).

**Figure 4. Palpation of porcine livers at a slaughterhouse meat control facility.**

**Figure 5. A presumptive mycobacterial lesion in a slaughtered pig liver.**
Visual detection is not sensitive or accurate (Hiller et al. 2013, Komijn et al. 2007, Wisselink et al. 2010). Similar lesions can be caused by other hazardous food safety microbes (Faldyna et al. 2012, Hamilton et al. 2002, Hiller et al. 2013, Komijn et al. 2007, Pavlik et al. 2003) and mycobacteria can also be isolated from lymph nodes that do not exhibit visible lesions (Offermann et al. 1999, Wisselink et al. 2006). The tuberculin test has been established as a screening method for the detection of mycobacteria-positive pigs prior to slaughter, but this method has low sensitivity (Faldyna et al. 2012, Francis et al. 1978). Therefore, there is a great need to develop new approaches for the detection of mycobacteriosis in pigs (Faldyna et al. 2012).

1.5. Serological response and monitoring of porcine mycobacterial infections

Several authors have suggested that *M. avium* may cause porcine infections and may be a potential food safety hazard for humans. Detection of mycobacterial disease in a live animal is often very difficult. Therefore, must the presence of disease be determined by post mortem examination. Infection in swine exposed to *M. avium* is usually associated with the lymph nodes of the head, liver and the digestive tract and rarely spreads to other locations. Caseous lesions can also be found in porcine kidneys and spleen (Hiller et al. 2013, Van Ingen et al. 2010, Offermann et al. 1999, Thoen et al. 2006), but the formation of lesions caused by *M. avium* infections may take several months in pigs (Wisselink et al. 2006). Ulceration and necrosis of the skin have also been observed in *M. avium* infections (Agdestein et al. 2012). Post mortem visual inspection of lymph nodes and livers can give a high number of both false positive and negative results because it is a non-specific and non-sensitive test respectively (Eisenberg et al. 2012, Faldyna et al. 2012, Hiller et al. 2013, Komijn et al. 2007, Wisselink et al. 2006, Wisselink et al. 2010). The sensitivity of visual meat inspection has been found to be highest in pigs infected by *M. avium* at an age of between 2.5 and 4.5 weeks, but is low in pigs infected at the age of 18 weeks (Wisselink et al. 2006). Mycobacterial infections without any visible lesions have been reported (Brown et al. 1979, Dvorska et al. 1999, Hiller et al. 2013, Offermann et al. 1999, Wisselink et al. 2006). Repeated infections by *M. avium* may cause an altered immune response and inhibit the formation of lesions in pigs. It has been reported that pigs that were experimentally infected three times had low number of lesions in their lymph nodes (Wisselink et al. 2006). In such a case mycobacteria-infected pigs can pass the visual post mortem inspection (Wisselink et al. 2006). Also other bacteria, such as *Rhodococcus*, have been isolated from pathological, mycobacteria-like lesions in pigs (Dvorska et al. 1999, Faldyna et al. 2012, Hiller et al. 2013, Komijn et al. 2007, Pavlik et al. 2003). Cross-contamination with other pathogens, for example salmonella (Hamilton et al. 2002), has also been reported. Up to two thirds of the reported granulomatous
malformations in porcine lymph nodes result for reasons other than mycobacterial infection (Hiller et al. 2013).

The tuberculin skin test is the standard method for diagnosis of mycobacterial infections in living animals, but the sensitivity of the test is low (Monagham et al. 1994, Stepanova et al. 2011). Immunological responses detected with the tuberculin skin test can be applied at herd level only and they are relatively inaccurate in that case (Eisenberg et al. 2012). Rather poor correlations were reported between results of the ELISA-test (Eisenberg et al. 2012), gamma interferon release assay (Faldyna et al. 2012) and the tuberculin skin test (Eisenberg et al. 2012, Faldyna et al. 2012).

Several methods based on serological response of mycobacterial infections have been published (Boadella et al. 2011, Faldyna et al. 2012). Immunological response, such as the interferon release assay, may be more sensitive than the tuberculin test, and the interferon assay can be used to diagnose \textit{M. avium} infections in live and naturally-infected pigs (Faldyna et al. 2012). \textit{M. avium} induced central memory cells in porcine infections, but a six month period at least was needed to detect cell-mediated immunity to \textit{M. avium} in pigs (Stepanova et al. 2011). This is problematic because most finisher pigs are slaughtered around the age of six months. Moreover, the \textit{in vitro} re-stimulation interferon gamma production was decreased (Stepanova et al. 2011). Some lymphocyte release may cause long-term immunity in pigs infected with \textit{M. avium} (Stepanova et al. 2011). Recently, several mycobacteria-specific tests were applied to describe the correlations among abortions, re-breedings or stillbirths and mycobacterial infections (Eisenberg et al. 2012.). Stepanova et al. (2011) concluded that interferon gamma and lymphocyte transformation may represent a specific method for the identification of individual \textit{M. avium} infections in pigs, but more detailed studies are needed (Stepanova et al. 2011). The results of Stepanova et al. (2011) indicate that the interferon gamma release assay and lymphocyte transformation test can, in some cases, be used for the identification of \textit{M. avium}-infected pigs (Stepanova et al. 2011).

Hiller et al. (2013) applied a mycobacterial-specific enzyme-linked immunosorbent assay (ELISA) to test for the presence of \textit{M. avium} antibodies in blood samples of slaughtered pigs in the Netherlands and Germany. The presence of \textit{M. avium} antibodies was detected to estimate the prevalence of mycobacterial infections at the herd level. The \textit{M. avium} ELISA test was validated to identify \textit{M. avium} positive farms. The validation results in this research showed that the sensitivity of an individual test was low and only 20% of the bacteriologically positive herds could be identified when 36 blood samples were tested. The low sensitivity at the herd level was supposed to be due to the presence of infections with other \textit{M. avium} serotypes that have reduced immunity towards the antigens tested. Closely related \textit{M. avium} subsp. \textit{avium} and subsp. \textit{hominissuis} showed different capacities to stimulate the porcine immune system. \textit{M. avium} subsp. \textit{hominissuis} showed low cell-mediated immunity with high individual variability (Dvorska et al. 2004, Hiller et al. 2013, Stepanova et al. 2012). However, Hiller et al. (2013) concluded that serological screening using a \textit{M. avium-
specific ELISA-test was able to identify bacteriological \textit{M. avium}-positive herds and pig populations at risk of \textit{M. avium} infections. The discriminatory power between infected and non-infected farms using the ELISA test could be improved with additional mycobacterial antigens (Hiller et al. 2013).

\textit{M. avium} subsp. \textit{hominissuis} is a weaker pathogen than \textit{M. avium} subsp. \textit{avium} (Faldyna et al. 2012). The developed commercial serological tests are not suitable for detecting \textit{M. avium} subsp. \textit{hominissuis} (Domingos et al. 2009, Faldyna et al. 2012). The immunological parameters of \textit{M. avium} subsp. \textit{avium} and \textit{M. avium} subsp. \textit{hominissuis} have been compared and the systemic immune response during experimental mycobacteriosis of pigs has been measured. Release of interferon gamma can be detected after five weeks in \textit{M. avium} subsp. \textit{avium} and subsp. \textit{hominissuis}-infected pigs, but not in every individual (Agdestein et al. 2012). The cell-mediated immunological response of \textit{M. avium} subsp. \textit{hominissuis} is significantly weaker when compared with that of \textit{M. avium} subsp. \textit{avium}. The release of mycobacterial specific antibodies or gamma interferon was low and variable in \textit{M. avium} subsp. \textit{hominissuis} infections compared with that in \textit{M. avium} subsp. \textit{avium} infections (Stepanova et al. 2012). The release of pro-inflammatory cytokines from macrophages was notably higher during \textit{in vitro} experiments when the inductor was \textit{M. avium} subsp. \textit{avium} than with \textit{M. avium} subsp. \textit{hominissuis}. In addition, the quantity of \textit{M. avium} subsp. \textit{hominissuis} was at least 1000 fold lower than that of \textit{M. avium} subsp. \textit{avium} in infected gastro-intestinal tissues of pigs (Slana et al. 2010, Stepanova et al. 2012). The macrophage response to \textit{M. avium} subsp. \textit{hominissuis} infections was reported to be significantly weaker than the response to \textit{M. avium} subsp. \textit{avium} infections \textit{in vitro}. \textit{M. avium} subsp. \textit{hominissuis}-infected macrophages also showed weaker induction of pro-inflammatory cytokines and chemokines (Stepanova et al. 2012). The immunological response also differed among different \textit{M. avium} subsp. \textit{hominissuis} genotypes (El-Sayed et al. 2013, Stepanova et al. 2012, Thegerström et al. 2012). It can be concluded that \textit{M. avium} subsp. \textit{hominissuis} can only induce a weak cell-mediated immunity. However in some cases, positive results were obtained for the interferon gamma release assay of \textit{M. avium} subsp. \textit{hominissuis} infections of pigs without detecting specific antibodies (Stepanova et al. 2012). The interferon gamma release assay may represent an effective tool for discrimination of \textit{M. avium} subsp. \textit{avium}-infected pigs, but be too inaccurate for detection of \textit{M. avium} subsp. \textit{hominissuis} infections (Stepanova et al. 2012).

Moreover, most of the immunological tests for mycobacterial infections in pigs have been applied to \textit{M. avium} subsp. \textit{avium}, but the majority of \textit{M. avium} strains in mycobacteriosis of pigs are \textit{M. avium} subsp. \textit{hominissuis} (Domingos et al. 2009, Garrido et al. 2010, Shitaye et al. 2006, Stepanova et al. 2011, Stepanova et al. 2012). In the porcine mycobacteriosis caused by \textit{M. avium} subsp. \textit{hominissuis} the infected pigs produced significantly lower levels of mycobacterial-specific antibodies and interferon gamma as compared with \textit{M. avium} subsp. \textit{avium}-infected pigs (Stepanova et al.
The immunodominant antigens in different mycobacterial species may not be cross-reactive (Faldyna et al. 2012) and therefore more reliable detection methods are required for the identification of mycobacterial infections in routine diagnosis of pigs (Faldyna et al. 2012, Wisselink et al. 2006).

2. Aims of the study

1. Examination and quantification of environmental samples from piggeries for the presence of mycobacteria in order to develop and apply DNA and rRNA-based methods for the detection of potentially hazardous mycobacteria in the piggery environment.

2. Sequencing (16S rDNA) and typing (RFLP, MIRU-VNTR) of the mycobacterial isolates of porcine origin and comparison of them with human isolates to examine the similarity of the \textit{M. avium} strains originating from slaughter pigs and human cases regarding public health aspects in Finland.

3. Parallel application of Restricted Fragment Length Polymorphism (RFLP) patterns and Variable-Number Tandem Repeat (VNTR) typing of genetic interspersed repetitive units of mycobacteria (MIRUs).

4. Quantification of \textit{Mycobacterium avium} subspecies in pig tissue material using real-time quantitative PCR.

3. Materials and methods

3.1. Samples and experimental design

3.1.1. Piggery environmental samples and experimental design

Piggery environmental samples were collected from birth to slaughter farms with high condemnation rates for environmental mycobacteria. All laboratory work was done according to strict hazard class two working standards. The total viable mycobacteria contents were analyzed from environmental samples taken from five piggeries, about 15-20 samples per piggery, totalling 94 samples, with 2 parallel samplings. Regard-
ing meat control, the prevalence of tuberculous lesions in the selected five piggeries was more than 4% during 2002-2004.

The experimental design for piggery environmental samples is shown in Fig. 6. The results were confirmed using 16S rRNA sandwich hybridization.

**Experimental design, piggery environmental samples**

![Experimental design diagram]

*Figure 6. Experimental design for piggery environmental samples.*

### 3.1.2. Pig organs and humans samples

Pig organ samples were collected in Finland from the slaughter line after meat inspection. 16 *M. avium*-positive pig organ samples from these were used (papers II and III). Seven additional samples of pig organs from the Netherlands were kindly provided by Gerard Wellenberg (paper IV).

Thirteen clinical *M. avium* isolates collected from Finnish human patients in 2001-2004 were randomly selected from the strain collection of the National Public Health Institute. The origin of the isolates was sputum, bronchial washings and lung biopsies (papers II and III).

*M. avium* strains from pigs and humans, in addition to reference strains, were stored at the culture collection of the Mycobacterial Reference Laboratory, The National Public Health Institute in Turku, Finland.
3.2. Typing of mycobacteria

Mycobacteria strains (16 pig and 13 human) were identified by direct sequence determination of 16S rRNA gene fragments and genotyped both with IS1245 restriction fragment length polymorphism (RFLP) and Variable-Number Tandem Repeat (VNTR) of genetic interspersed repetitive units of mycobacteria (MIRUs). Identification and RFLP and MIRU-VNTR typing were carried out in the mycobacterial reference laboratory, National Public Health institute in Turku, Finland. The discriminatory indices (DI) of RFLP and MIRUs were calculated.

3.3. Mycobacteria specific real-time qPCR

Description of the samples in real-time qPCR is shown in Table 1. Samples were kindly provided by G. Wellenberg.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig 4, sample 1.</td>
<td>Lesion</td>
</tr>
<tr>
<td>Pig 4, sample 2.</td>
<td>Outside the lesion</td>
</tr>
<tr>
<td>Pig 9-5577, sample 1.</td>
<td>Lesion</td>
</tr>
<tr>
<td>Pig 9-5577, sample 2.</td>
<td>Outside the lesion</td>
</tr>
<tr>
<td>Pig Austria 3, sample 1.</td>
<td>Lesion</td>
</tr>
<tr>
<td>Pig Austria 3, sample 2.</td>
<td>Outside the lesion</td>
</tr>
<tr>
<td>Pig 187, sample 1.</td>
<td>Lesion</td>
</tr>
<tr>
<td>Pig 187, sample 2.</td>
<td>Outside the lesion</td>
</tr>
</tbody>
</table>

*Table 1. Samples for mycobacterial quantification using qPCR.*

Isolation of mycobacterial DNA from tissue specimens was originally attempted according to a standard protocol, but the quantity of isolated mycobacterial DNA was low. Therefore, the protocol was modified to increase the mycobacterial cell wall lysis by digesting the tissue at 65°C with Proteinase K under agitation at 160 rpm for 16 h. This novel modification improved markedly the amount of isolated mycobacterial DNA. The detailed protocol is described in paper IV.
4. Results and discussion

4.1. Viable mycobacteria in piggeries

Mycobacterial growth was found in all bedding materials: sawdust, straw, peat and wood chips in most cases, water and food samples in many cases, but rarely in dust and spider webs. (Fig. 7(A&B), 8(A&B), 9(A&B), 10(A&B), 11(A,B&C)), (Tab. 2).

Figure 7 (A&B). Mycobacterial growth was found in over 60% of used bedding material samples inside the piggeries.

Figure 8 (A&B). Mycobacterial growth was found in around 35% of unused bedding material samples outside the piggeries.
Figure 9 (A&B). Mycobacteria grew in around 25% of feed samples inside the piggeries.

Figure 10 (A&B). Mycobacteria grew in approximately 20% of water samples inside piggery.
Figure 11 (A, B&C). No mycobacterial growth was found within eight weeks of cultivation in ventilation dust, colostrum or spider webs, but some growth was registered in spider webs and ventilation dust after three months of cultivation.

<table>
<thead>
<tr>
<th>Sample group description, (figure)</th>
<th>Result, mycobacterial growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Used bedding inside piggery (7, A&amp;B)</td>
<td>&gt;60 % of samples</td>
</tr>
<tr>
<td>Unused bedding outside piggery (8, A&amp;B)</td>
<td>appr. 35 % of samples</td>
</tr>
<tr>
<td>Pig feed inside the piggery (9, A&amp;B)</td>
<td>appr. 25 % of samples</td>
</tr>
<tr>
<td>Pig drinking water inside the piggery (10, A&amp;B)</td>
<td>appr. 20 % of samples</td>
</tr>
<tr>
<td>Ventilation dust and spider web (11, A&amp;C)</td>
<td>8 weeks negative, 3 months some</td>
</tr>
<tr>
<td>Colostrum (11B)</td>
<td>negative in 8 weeks and 3 months</td>
</tr>
</tbody>
</table>

Table 2. Mycobacterial growth in piggery samples.

Concentration of the mycobacteria in pig environmental samples was $10^5$ to $10^7$ in unused and $10^8$ cells per gram of dry weight at its highest in used bedding materials when *Mycobacterium*-specific hybridization probes were used for detection. Mycobacterial strains contain usually one or two 2 16S rRNA gene copies per cell (Klappenhbach et al. 2001, Pakarinen 2008). Since rRNA is found mainly in living cells, the results confirm that mycobacteria are viable and proliferate in bedding materials.
during their use in the pig-rearing environment.

The routes of infection for environmental mycobacteria are unclear, but several previously publications reported that bedding materials may be the source of infection in pigs (Matlova et al. 2003, Matlova et al. 2004, Matlova et al. 2005, Pakarinen 2008, Windsor et al. 1984) and mycobacteria from farm originating faecal materials can be found in drinking water (Bland et al. 2005, Pakarinen 2008). Quantitative methods are required for the detection of the high concentrations of potentially infective mycobacteria in the environment (Nichols et al. 2004, Pakarinen 2008). In this work we quantified mycobacterial concentration from piggery bedding materials, something that had not been done earlier.

4.2. Typing of mycobacteria by IS1234 RFLP and MIRU-VNTR methods

*M. avium* isolates obtained from pig livers and clinical human samples were compared using the IS1245 RFLP analysis to evaluate the similarity between the strains of human and swine origin. Nearly identical IS1245 RFLP patterns were found from *M. avium* isolates from pigs and humans. The similarity was confirmed using MIRU-VNTR analysis (Fig. 12).

![Figure 12. M. avium isolates MIRU-VNTR types and their diversity in IS 1245 RFLP analysis.](image-url)
One human and one porcine strain clustered identically using both typing methods, indicating that the strains were clonal. The same \textit{M. avium} strains are able to infect both humans and pigs. Our results concerning the high degree of similarity between \textit{M. avium} subsp. \textit{hominissuis} of human and porcine origins are in agreement with those of other studies (Komijn et al. 1999, Möbius et al. 2006, Thorel et al. 2001). Similar RFLP-profiles for \textit{M. avium} were also found in peat, human and swine samples (Agdestein et al. 2011, Bauer et al. 1999, Johansen et al. 2009, Matlova et al. 2005), supporting the hypothesis that human and pig infections derive from the same source in the environment. Their results are in accordance with those of other studies (Johansen et al. 2007, Johansen et al. 2009, Möbius et al. 2006, O’Grady et al. 2000).

Four different mycobacteria strains were simultaneously found in one pig, which could be the result of a heavy load of \textit{M. avium} in the piggeries or high susceptibility of some pigs to \textit{M. avium} infections. Different \textit{M. avium} strains in one pig were also reported by Eisenberg et al. (2010). Our study showed that piggery environments could be important sources of mycobacterial infections for pigs and humans (Pakarinen et al. 2007). This is in accordance with several published reports (Arbeit et al. 1993, Oliveira et al. 2000, Pate et al. 2008, Agdestein et al. 2011, Agdestein et al. 2014).

The 16S rRNA sequences are similar in \textit{M. avium} strains from humans and pigs. They grouped together using different typing methods and are classified as \textit{M. avium} subsp. \textit{hominissuis} (Mijs et al. 2002). The IS1245 insertion sequence is specific to \textit{M. avium} subsp. \textit{hominissuis} and those strains have a high degree of IS1245-based polymorphism, which can be used to detect the genetic diversity among \textit{M. avium} strains, (Guerrero et al. 1995, de Sequeira et al. 2005, Johansen et al. 2007, Eisenberg et al. 2012, El-Sayed et al. 2013).

Genotyping \textit{M. avium} isolates has been done many times previously using RFLP (El-Sayed et al. 2013, Moravkova et al. 2008) and MIRU-VNTR typing (Despierres et al. 2012, El-Sayed et al. 2013, Pate et al. 2011, Romano et al. 2005). In our studies the major RFLP clusters grouped together with MIRU-VNTR clusters. IS1245 RFLP patterns and MIRU-VNTR mostly resulted interspecies rather than intraspecies clusters. The discriminatory index for IS1245 RFLP was 0.98 and was 0.92 for MIRU-VNTR typing. Our results from the Finnish isolates are parallel with other publications (Eisenberg et al. 2012, Inagaki et al. 2009, Pate et al. 2011). RFLP and MIRU-VNTR typing resulted in high levels of reproducibility and genetic diversity. RFLP and MIRU-VNTR typing methods show great potential for epidemiological mapping and determination of transmission pathways for \textit{M. avium} subspecies (Eisenberg et al. 2012, El-Sayed et al. 2013).
4.3. Quantification of *Mycobacterium* subspecies in pig tissues using real-time quantitative PCR.

*M. avium* subspecies in pig tissues are difficult, and sometimes impossible, to quantify using culture methods. To date there have been very few reports on the identification of *M. avium* strains directly from infected tissue without previous culture (Agdestein et al. 2011, Slana et al. 2010, Tell et al. 200). In this work we developed and applied culture-independent real-time quantitative PCR assays for detecting *M. avium* strains in pig tissues. Concentrations of mycobacteria cells per gram in organ tissues, with or without lesions, were from about $10^5$. Similar results were reported earlier (Agdestein et al. 2011). The response of the qPCR assay to the logarithmic quantity of *M. avium* added to pig liver was linear, approximately in the range of $10^5$ to $10^7$ bacteria per gram (Fig. 13). The qPCR method results were confirmed with microscopy calculation. Recently several qPCR methods have been developed for the detection of mycobacteria strains from human, animal and environmental samples. However, less laborious and complex methods are needed (Kriz el al 2014). In this work we developed an accurate qPCR method for identifying mycobacterial infections in pigs. Our protocol provides a novel, efficient and simple protocol to detecting total mycobacterial cell numbers, including for samples from tissues lacking visible lesions.

![Figure 13. Response of the Mycobacterium-specific qPCR assay to *M. avium* subspecies avium ATCC 25291 cells in pig liver. Mycobacterial DNA was quantified in five parallel extracts of pig liver spiked with *M. avium* subspecies avium (1 x $10^4$ to 1 x $10^7$ bacteria per gram). Open symbols denote qPCR results below the detection limit (4 x $10^4$ mycobacteria per gram). The figure shows that the dispersion of the number of gene copies per gram for the parallel samples decreases when the number of colony forming units (CFU) increases.](image)
5. Conclusions

Environmental mycobacteria are regarded as a potential zoonotic risk and cause economic losses worldwide. The results reported in this thesis show that:

1. Viable mycobacteria commonly occur in piggeries and can multiply in bedding materials, reaching concentrations that can represent a potential infection risk for humans and animals.

2. Identical *M. avium* subsp. *hominissuis* genotypes were obtained from human and porcine isolates, suggesting that the bacterium is transmitted between pigs and humans, or that pigs and humans share common environmental sources of infection.

3. Several mycobacteria strains found in a single pig may be the result of a heavy load of mycobacteria in the piggery environment, or susceptibility of some pigs to mycobacteria infections.

4. The real-time qPCR method developed was suitable for the identification of tuberculous infections of pigs, including those without visible lesions.

Implications
Future research on mycobacterial infections and epidemiology are needed to estimate the common sources and reservoirs of mycobacteria. However, the epidemiology of mycobacteria is very complex and challenging.

6. Acknowledgements

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